Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 12255–12259, December 1995
Microbiology

The sigma factor σ^S affects antibiotic production and biological control activity of *Pseudomonas fluorescens* Pf-5

(pyrrolnitrin/pyoluteorin/2,4-diacetylphloroglucinol/stationary phase/biocontrol/*rpoS* gene)

ALAIN SARNIGUET*, JENNIFER KRAUS†, MARCELLA D. HENKELS†, ANDREA M. MUEHLCHEN†, AND JOYCE E. LOPER†‡

*Institut National de la Recherche Agronomique, Station de Pathologie Végétale, Centre de Recherches de Rennes, BP 29, 35650 Le Rheu, France; and †U.S. Department of Agriculture, Agricultural Research Service, Horticultural Crops Research Laboratory, 3420 Northwest Orchard Avenue, Corvallis, OR 97330

Communicated by R. James Cook, Washington State University, Pullman, WA, September 21, 1995

ABSTRACT ***Pseudomonas fluorescens* Pf-5, a rhizosphere-inhabiting bacterium that suppresses several soilborne pathogens of plants, produces the antibiotics pyrrolnitrin, pyoluteorin, and 2,4-diacetylphloroglucinol. A gene necessary for pyrrolnitrin production by Pf-5 was identified as *rpoS*, which encodes the stationary-phase sigma factor σ^S. Several pleiotropic effects of an *rpoS* mutation in *Escherichia coli* also were observed in an RpoS$^-$ mutant of Pf-5. These included sensitivities of stationary-phase cells to stresses imposed by hydrogen peroxide or high salt concentration. A plasmid containing the cloned wild-type *rpoS* gene restored pyrrolnitrin production and stress tolerance to the RpoS$^-$ mutant of Pf-5. The RpoS$^-$ mutant overproduced pyoluteorin and 2,4-diacetylphloroglucinol, two antibiotics that inhibit growth of the phytopathogenic fungus *Pythium ultimum*, and was superior to the wild type in suppression of seedling damping-off of cucumber caused by *Pythium ultimum*. When inoculated onto cucumber seed at high cell densities, the RpoS$^-$ mutant did not survive as well as the wild-type strain on surfaces of developing seedlings. Other stationary-phase-specific phenotypes of Pf-5, such as the production of cyanide and extracellular protease(s) were expressed by the RpoS$^-$ mutant, suggesting that σ^S is only one of the sigma factors required for the transcription of genes in stationary-phase cells of *P. fluorescens*. These results indicate that a sigma factor encoded by *rpoS* influences antibiotic production, biological control activity, and survival of *P. fluorescens* on plant surfaces.**

Fluorescent pseudomonads are ubiquitous inhabitants of plant surfaces, including roots, leaves, and floral parts, and certain strains are promising agents for the biological control of plant diseases caused by phytopathogenic fungi and bacteria. The capacity of these bacteria to establish populations on root surfaces of plants and produce antibiotics that inhibit fungal and bacterial phytopathogens contributes to their effectiveness in suppressing soilborne plant diseases (1, 2). Antibiotic production (3) and the ability to survive exposure to environmental and physiological stresses (4, 5), such as those encountered by microorganisms on plant surfaces, are characteristics of stationary-phase cells of *Pseudomonas* spp. Although little is known about the physiological status of bacteria inhabiting natural environments, it is likely that bacteria exist for the majority of their lives in a state of starvation (6), perhaps analogous to the stationary phase of bacteria in culture. Bacterial cells undergo complex physiological and morphological changes upon entry into stationary phase, which is defined by the cessation of exponential growth imposed by nutrient limitation or other factors (7).

Pseudomonas fluorescens strain Pf-5, when inoculated onto seeds at the time of planting, establishes in the spermosphere and rhizosphere, where it suppresses the soilborne fungi *Rhizoctonia solani* and *Pythium ultimum* (8, 9). These fungi can infect seeds and roots, thereby reducing seedling emergence and survival. Strain Pf-5 also colonizes wheat straw residue, where it suppresses formation of ascocarps by the fungus *Pyrenophora tritici-repentis* (10), which causes the leaf disease known as tan spot of wheat. Pf-5 produces the antifungal antibiotics pyrrolnitrin (8), pyoluteorin (9), and 2,4-diacetylphloroglucinol (Phl) (11). Each antibiotic has a unique spectrum of activity against fungal pathogens inhibited by Pf-5: pyrrolnitrin inhibits *R. solani* (8) and *Pyrenophora tritici-repentis* (10), pyoluteorin inhibits *Pythium ultimum* (9), and Phl inhibits all three fungi (11–13). Antibiotic production by *P. fluorescens* is under global genetic control by a two-component regulatory system composed of the sensor protein ApdA (also called LemA) (ref. 14; S. T. Lam and T. D. Gaffney, personal communication) and the response regulator GacA (15, 16). A mutation in *apdA* or *gacA* of *P. fluorescens* abolishes the production of pyrrolnitrin, pyoluteorin, Phl, hydrogen cyanide, extracellular protease(s), and tryptophan side-chain oxidase (14–16).

We have characterized a regulatory gene influencing the capacity of *P. fluorescens* Pf-5 to produce antibiotics, tolerate stresses imposed by H_2O_2 or salt, persist on certain plant surfaces, and suppress specific plant diseases. The nucleotide sequence and deduced amino acid sequence§ of the gene are similar to those of the *rpoS* gene of *Escherichia coli* and its protein product σ^S (also called σ^{38}), a sigma factor that controls the transcription of many genes expressed in response to starvation and during the transition to stationary phase (7, 17). On the basis of sequence similarity to the *rpoS* genes of *Pseudomonas aeruginosa* (18) and *E. coli* (19), and the pleiotropic phenotype conferred by the locus, we identified the gene in *P. fluorescens* Pf-5 as *rpoS*.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions. *P. fluorescens* JL3985, obtained after Tn*5* mutagenesis of Pf-5 (10), is a pleiotropic mutant that does not produce pyrrolnitrin, overproduces pyoluteorin and Phl (previously called antibiotic 3), and is less able to colonize wheat straw relative to the parental strain Pf-5. Strain JL3985 is prototrophic and its exponential growth rate in culture is similar to that of Pf-5 (10). Plasmid pJEL5649 contains a 2.9-kb *Eco*RI fragment of wild-type DNA corresponding to the region mutagenized in strain JL3985. pJEL5649 was constructed by inserting the 2.9-kb *Eco*RI fragment from pJEL1884 (10) into the plasmid

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: Phl, 2,4,-diacetylphloroglucinol; cfu, colony-forming units; ORF, open reading frame.

‡To whom reprint requests should be addressed.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. U34203).

vector pJEL01, which confers tetracycline resistance and is stably maintained in *P. fluorescens*. pJEL01 was derived by inserting the 9.4-kb *Bam*HI fragment of pVS1, which carries regions required for mobilization, replication, and stable maintenance in *Pseudomonas* spp. (20), into the unique *Bcl* I site of pACYC184, which has a p15A replication origin (21) and carries genes for resistance to tetracycline and chloramphenicol. *P. fluorescens* and *E. coli* were cultured as described previously (10, 22).

DNA Manipulations and Nucleotide Sequencing. Plasmids were purified with Promega or Qiagen (Chatsworth, CA) kits. Gel electrophoresis, ligations, and transformations were performed by using standard methods (23). Plasmids pJEL01 and pJEL5649 were mobilized into *P. fluorescens* from *E. coli* strain HB101 (23) by using the helper plasmid pRK2013, as described previously (10).

Initial sequencing of the *rpoS* locus was performed on regions flanking the Tn*5* insert of JL3985 (10) with an oligonucleotide primer complementary to bases 37–18 in the inverted repeat of Tn*5* (5′-GGTTCCGTTCAGGACGCTAC-3′) (24). Sequencing within the 2.9-kb *Eco*RI fragment cloned in pJEL5649 was directed by oligonucleotide primers designed from previous sequence determinations of the *rpoS* region. DNA sequence analysis and comparisons with sequences contained in the GenBank and European Molecular Biology Laboratory data bases were done with software from the Genetics Computer Group (Madison, WI).

Assessment of Secondary Metabolite and Exoenzyme Production. Antibiotics were extracted from both cells and spent media of cultures grown for 2 days in 5 ml of 523 medium (25) at 27°C with shaking (200 rpm). After centrifugation (5000 × *g*, 5 min) of cultures, the bacterial pellet was suspended in 5 ml of acetone, and the suspension was sonicated in an ultrasonic cleaner for 30 sec. Cell suspensions were centrifuged (10,000 × *g*, 10 min) and the acetone supernatant was removed and dried under reduced pressure. Culture supernatants were adjusted to pH 2.0 with 1 M HCl and extracted three times with 2 ml of ethyl acetate. The organic phases were combined and dried under reduced pressure. Samples redissolved in 100 μl of methanol were analyzed by C_{18} reverse-phase high-performance liquid chromatography [0.8 × 10 cm Waters Nova-pak radial compression cartridge; 45% water/30% acetonitrile/25% methanol (vol/vol); 1.5 ml/min]. Antibiotics were detected by UV spectroscopy and quantified against authentic standards (pyrrolnitrin, λ = 225 nm, retention time t_r = 10.2 min; pyoluteorin, λ = 310 nm, t_r = 3.4 min; Phl, λ = 278 nm, t_r = 6.4 min). Total antibiotic concentrations in cultures of *P. fluorescens* were calculated as the sum of concentrations in cells and culture supernatants.

Assays for detection of tryptophan side-chain oxidase (26), hydrogen cyanide (27), and extracellular protease (28) production were assessed visually.

Stress Response. Methods for determining response of *P. fluorescens* to stresses imposed by H_2O_2 or NaCl were adapted from those used to determine the response of *E. coli* to these compounds (29). Cultures were grown in minimal medium M9 (23) supplemented with 0.4% (wt/vol) glucose at 27°C with shaking (200 rpm). Exponential-phase cells [≈3 × 10^8 colony-forming units (cfu)/ml] were obtained from 5-ml cultures in test tubes (18 × 150 mm) after 6-hr incubation. Stationary-phase cells (≈3 × 10^9 cfu/ml) were obtained from similar cultures 4 hr after the optical density (λ = 600 nm) of cultures stopped increasing (i.e., the cultures entered stationary phase). Cultures were centrifuged (5000 × *g*, 3 min) and cells were washed twice in M9 medium. Exponential-phase and stationary-phase cells prepared as above were suspended in 5 ml of M9 medium or 5 ml of M9 medium containing either 15 mM H_2O_2 or 2.4 M NaCl. The resulting cell suspensions (≈3 × 10^8 cfu/ml) were incubated with shaking at 27°C, and cell culturability was determined by spreading samples of appropriate dilutions on Luria–Bertani medium (23). Three replicate cultures were evaluated for each treatment, and the experiment was done twice.

Biological Control of *Pythium* Damping-off of Cucumber. Inoculum of *P. fluorescens* was grown in King's medium B broth for 22 h at 27°C with shaking (200 rpm) and bacterial cells were washed in sterile water (22). Cucumber (*Cucumis sativus* cv. Marketmore) seeds were soaked for 10 min in aqueous cell suspensions of *P. fluorescens* to obtain 10^8 cfu per seed and planted individually in pots containing 20 ml of pasteurized soil (Newberg fine sandy loam adjusted to a soil matric potential of −0.01 MPa by adding water), which had been infested with *P. ultimum* as described previously (22). Pots were maintained in growth chambers (20°C, 12 h photoperiod). Ten days after planting, the numbers of surviving and dead seedlings were recorded, and percent seedling emergence and percent seedling survival were compared by using χ^2 analysis. Treatment groups were composed of 100 replicate pots, and the experiment was done twice. Results from both experiments were similar, and only data from the first experiment are presented.

Population Size of *P. fluorescens* on Plant Surfaces. Cucumber seeds were treated with *P. fluorescens* (≈10^4 or ≈10^8 cfu per seed) and planted in individual pots containing 70 ml of nonpasteurized soil, and the pots were incubated as described for the biological control experiments. Periodically, 10 individual seeds (0, 6, 12, 24, and 48 h after planting) or emerged seedlings (4, 6, and 10 days after planting) were harvested from each treatment. Population sizes of Pf-5 and JL3985, which are naturally resistant to streptomycin and ampicillin, were estimated by dilution-plating of seed or seedling washings on King's medium B containing streptomycin (50 μg/ml) and ampicillin (100 μg/ml) as described previously (22). The results from two independent experiments were similar and only data from the first experiment are presented.

RESULTS

Secondary Metabolite and Enzyme Production by a Pleiotropic Mutant of *P. fluorescens* Pf-5. Strain Pf-5 produced pyrrolnitrin and pyoluteorin when grown in 523 medium (Table 1). Although Pf-5 produces Phl in certain culture media (11), no Phl was detected in supernatants of Pf-5 cultures grown in 523 medium (Table 1). The Tn*5*-insertion mutant JL3985 produced no detectable pyrrolnitrin. Plasmid pJEL5649, which contains a 2.9-kb *Eco*RI fragment of Pf-5 genomic DNA cloned in pJEL01, restored pyrrolnitrin production to JL3985. The restored strain JL3985(pJEL5649) produced slightly more pyrrolnitrin than Pf-5, possibly because of the copy number (four to nine copies per cell) of pVS1-derived vectors in *Pseudomonas* spp. (20). The mutant JL3985 overproduced pyoluteorin and Phl compared with levels produced by Pf-5; the restored strain JL3985(pJEL5649) produced only trace amounts of pyoluteorin and Phl.

Due to the pleiotropic effect of the Tn*5* insertion of JL3985 on antibiotic production, we evaluated other phenotypes that are controlled coordinately with antibiotic production by the

Table 1. Antibiotic production by *P. fluorescens* Pf-5 and derivative strains

Strain	Antibiotic conc., μg/ml of culture medium*		
	Pyrrolnitrin	Pyoluteorin	Phl
Pf-5	2.2 ± 0.1	6.6 ± 0.1	<0.1
JL3985	<0.1	37.7 ± 1.1	52.2 ± 3.2
JL3985(pJEL5649)	2.7 ± 0.2	0.1 ± 0.1	0.1 ± 0.1

*Concentrations of antibiotics were determined from extracts of bacterial cultures grown in 5 ml of 523 medium at 27°C for 48 hr. Values are mean ± SD of two independent experiments.

apdA gene of *P. fluorescens* Pf-5 (14). Mutant JL3985 exhibited no detectable tryptophan side-chain oxidase activity, whereas Pf-5 and JL3985(pJEL5649) expressed tryptophan side-chain oxidase activity. The pleiotropic mutation had no discernable effect on production of hydrogen cyanide or extracellular protease(s).

A Locus with Pleiotropic Effects on Secondary Metabolite Production by *P. fluorescens* Pf-5 Is Similar to the *rpoS* Gene of *E. coli*. An open reading frame (ORF) of 1008 bases followed by a rho-independent prokaryotic terminator sequence was identified within the 2.9-kb *Eco*RI fragment cloned in pJEL5649. The Tn*5* insertion of JL3985 was at nucleotide 398 of the ORF, such that the guanosine at the terminus of Tn*5* generated a *Bam*HI site (5′-GGATCC-3′). This *Bam*HI site was previously associated with the transposition of Tn*5* into the genome of JL3985 (10).

The DNA sequence of the 1008-bp ORF exhibited 80% identity with a putative *rpoS* gene from *P. aeruginosa* (1005 bases) (18) and 63% identity with an *rpoS* gene of *E. coli* (1029 bases) (19). The predicted amino acid sequence of the ORF is 88% identical to RpoS of *P. aeruginosa* and 64% identical to σ^S, the product of the *rpoS* gene of *E. coli* (Fig. 1). The 264 amino acids contained within the most highly conserved regions [1.2 through 4.2 (30)] are 95% and 77% identical to the corresponding regions of RpoS of *P. aeruginosa* and *E. coli*, respectively (Fig. 1). On the basis of nucleotide and predicted amino acid sequences, we hereafter refer to the ORF as the *rpoS* gene of Pf-5.

```
    1
Pf  ......MALS KEVPEFDIDD EVLLMESSID MDSMSNNEGA TPPSVRTKSK
Pa  ......MALK KEGPEFDHDD EVLLLEPGIM LDESSADEQP SPRAT.PKAT
Ec  MFRQGITGRS HLMSQNTLKV HDLNEDAEFD ENGVEVLDEK ALVEQEPSDN

    51                          1.2
    SSASLKQHKY IDYTRALDAT QLYLNEIGFS PLLSPEEEVH FARLSQSGDP
    TSFSSKQHKH IDYTRALDAT QLYLNEIGFS PLLTPEEEVH FARLAQKGDP
    DLAEEELLSQ GATQRVLDAT QLYLGEIGYS PLLTAEEEVY FARRALRGDV

    101       2.1                 2.2
    AGRKRMIESN LRLVVKIARR YVNRGLSLLD LIEEGNLGLI RAVEKFDPER
    AGRKRMIESN LRLVVKIARR YVNRGLSLLD LIEEGNLGLI RAVEKFDPER
    ASRRRMIESN LRLVVKIARR YGNRGLALLD LIEEGNLGLI RAVEKFDPER

    151  2.3          2.4                3.1
    GFRFSTYATW WIRQTIERAI MNQTRTIRLP IHVVKELNVY LRAARELTQK
    GFRFSTYATW WIRQTIERAI MNQTRTIRLP IHVVKELNVY LRAARELTHK
    GFRFSTYATW WIRQTIERAI MNQTRTIRLP IHIVKELNVY LRTARELSHK

    201                 3.1
        ••••••••••••••••••••••
    LDHEPSPEEI ANLLEKPVAE VKRMLGLNER VSSVDVSLGP DSDKTLLDTL
    LDHEPSPEEI ANLLEKPVAE VKRMLGLNER VTSVDVSLGP DSDKTLLDTL
    LDHEPSAEEI AEQLDKPVDD VSRMLRLNER ITSVDTPLGG DSEKALLDIL

    251 3.2                    4.1
                                    ••
    TDDRPTDPCE LLQDDDLSQS IDQWLSELTE KQREVVVRRF GLRGHESSTL
    TDDRPTDPCE LLQDDDLSES IDQWLTELTD KQREVVIRRF GLRGHESSTL
    ADEKENGPED TTQDDDMKQS IVKWLFELNA KQREVLARRF GLLGYEAATL

    301          4.2                          343
    •••••••••••••••••••••••
    EDVGLEIGLT RERVRQIQVE GLKRLREILE KNGLSSESLF Q*.
    EEVGQEIGLT RERVRQIQVE ALKRLREILE KNGLSSDALF Q*.
    EDVGREIGLT RERVRQIQVE GLRRLREILQ TQGLNIEALF RE*
```

FIG. 1. Predicted amino acid sequence of RpoS of *P. fluorescens* Pf-5 (*Pf*) aligned with the sequences of *P. aeruginosa* (*Pa*) and *E. coli* (*Ec*). Amino acids that are identical in all three sequences are indicated by highlighting. Dotted lines above the amino acid sequence indicate a predicted helix–turn–helix motif. Solid lines indicate regions conserved among sigma factors (30). Certain of these have been assigned putative functions: 2.1, binding to core polymerase; 2.3, DNA strand opening (with 2.1); 2.4, recognition of the −10 promoter region; 3.1, DNA binding; 3.2, binding to the core polymerase; 4.1 and 4.2, recognition of the −35 promoter region.

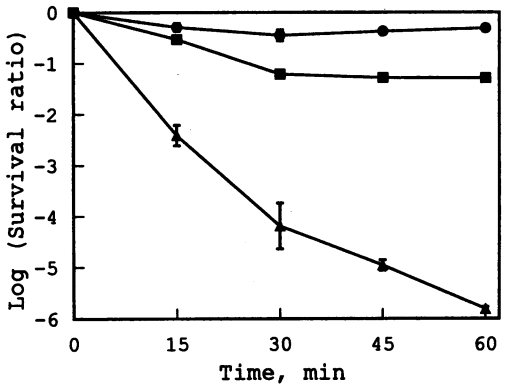

FIG. 2. Survival of stationary-phase cells of *P. fluorescens* Pf-5 (■), RpoS⁻ mutant JL3985 (▲), and restored mutant JL3985(pJEL5649) (●) during exposure to 15 mM H_2O_2. Numbers of culturable cells were determined by spreading samples of serial dilutions on Luria–Bertani medium. Survival was expressed as the $\log_{10}$ of the ratio of culturable cells in the exposed suspension to the number of culturable cells in the initial suspension. Error bars represent the standard deviation.

Stress Response of Stationary-Phase Cells of *P. fluorescens* Was Affected by *rpoS*. In *E. coli*, *rpoS* confers pleiotropic effects on stationary-phase cells, including tolerance of stresses imposed by hydrogen peroxide or high salt concentrations (29). Stationary-phase cells of *P. fluorescens* Pf-5 were similarly tolerant of H_2O_2, whereas stationary-phase cells of JL3985 lost culturability soon after exposure to 15 mM H_2O_2 (Fig. 2). Introduction of pJEL5649, which contains the wild-type *rpoS* gene, restored the capacity of stationary-phase cells of the RpoS⁻ mutant JL3985 to survive exposure to hydrogen peroxide. In addition, stationary-phase cells of Pf-5 or JL3985-(pJEL5649) were relatively tolerant of 2.4 M NaCl, whereas stationary-phase cells of JL3985 lost culturability after exposure to this salt concentration (Fig. 3). Stationary-phase cells of Pf-5 were more tolerant of 15 mM H_2O_2 or 2.4 M NaCl than were cells in exponential phase (data not shown).

Biological Control Activity and Population Size of *P. fluorescens* Pf-5 on Surfaces of Cucumber Seedlings Were Affected by *rpoS*. Pf-5 and its RpoS⁻ derivative strain JL3985 suppressed pre-emergence damping-off caused by *Pythium ultimum* to a similar extent, based on the percentage of seedlings that emerged from the soil (Table 2). In contrast, the RpoS⁻ mutant was superior to Pf-5 in suppression of postemergence

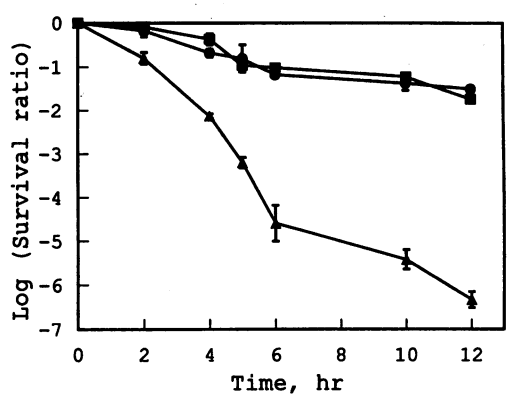

FIG. 3. Survival of stationary-phase cells of *P. fluorescens* Pf-5 (■), RpoS⁻ mutant JL3985 (▲), and the restored mutant JL3985-(pJEL5649) (●) during exposure to 2.4 M NaCl. Numbers of culturable cells were determined by spreading samples of serial dilutions on Luria–Bertani medium. Survival was expressed as the $\log_{10}$ of the ratio of culturable cells in the exposed suspension to the number of culturable cells in the initial suspension. Error bars represent the standard deviation.

Table 2. Suppression of *Pythium* damping-off of cucumber by *P. fluorescens*

Seed treatment*	Seedling emergence,† %	Seedling survival,‡ %
Pf-5	78[b]	61[b]
JL3985	86[b]	77[a]
None	39[c]	33[c]

*Bacterial cells were applied at 2×10^8 cfu per cucumber seed.
†Percent seedling emergence was calculated by dividing the number of seedlings that emerged from the soil by the number of seeds planted and multiplying by 100. Values within a column followed by a common letter do not differ significantly ($P = 0.05$) as determined by χ^2 comparisons.
‡Percent seedling survival was calculated by dividing the number of seedlings that were alive 10 days after planting by the number of seeds planted and multiplying by 100. Values within a column followed by a common letter do not differ significantly ($P = 0.05$) as determined by χ^2 comparisons.

damping-off of cucumber caused by *Pythium ultimum*, assessed as the percentage of surviving seedlings at 10 days after planting. When seeds were coated with high densities of bacterial cells ($\approx 10^8$ cfu per seed), the population size of Pf-5 was stable over the 10-day duration of the experiment, whereas the population size of the RpoS$^-$ mutant declined (Fig. 4*A*). When seeds were coated with low densities of bacterial cells ($\approx 10^4$ cfu per seed), however, the population sizes of Pf-5 and JL3985 increased at comparable rates and did not differ significantly over the course of the experiment (Fig. 4*B*).

DISCUSSION

Gram-negative bacteria, in response to starvation or upon entry to stationary phase, undergo a process of differentiation that leads to the development of a cellular state with markedly enhanced tolerance of a variety of individual stresses (7, 17). The stationary-phase sigma factor σ^s plays a critical role in this differentiation process (7, 17). A mutation in *rpoS* of *E. coli* has pleiotropic effects on cells in a state of starvation or during the transition into stationary phase, including a diminished capacity to survive exposure to environmental stresses (29). In this study, we demonstrated that a mutation in the *rpoS* gene of *P. fluorescens* Pf-5 similarly reduced the ability of the bacterium to tolerate stresses imposed by hydrogen peroxide and high salt concentrations; introduction of a wild-type *rpoS* gene restored stress tolerances of the RpoS$^-$ mutant to wild-type levels.

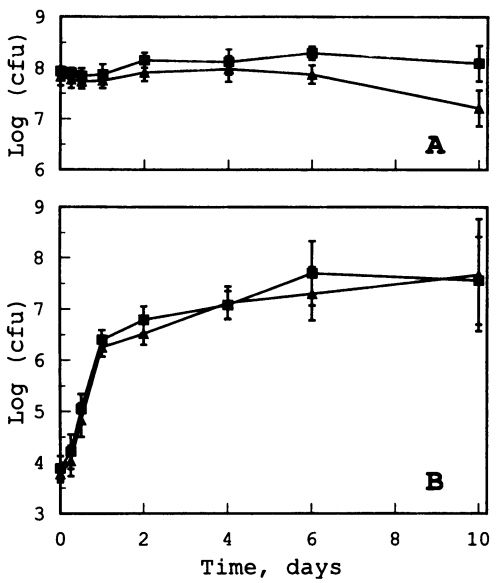

FIG. 4. Population sizes of *P. fluorescens* Pf-5 (■) and RpoS$^-$ mutant JL3985 (▲) on the surfaces of cucumber seeds (0–2 days) or seedlings (4–10 days). Seeds were inoculated with $\approx 10^4$ cfu per seed (*A*) or $\approx 10^8$ cfu per seed (*B*). Error bars represent the standard deviation.

Only a subset of the secondary metabolites and exoenzymes produced by *P. fluorescens* Pf-5 at the onset of, or during, stationary phase was affected by the *rpoS* mutation. Therefore, sigma factors other than σ^s are required for transcription of certain genes expressed by *P. fluorescens* Pf-5 during stationary phase. In *E. coli*, at least three sigma factors (σ^{70}, σ^{32}, and σ^s) are involved in gene expression during stationary phase (31). The RNA polymerase holoenzyme in which σ^s is the sigma factor binds to promoters of a subset of genes expressed preferentially by cells in stationary phase (32, 33). Other genes under control by *rpoS* appear to be affected indirectly, presumably by the products of genes directly transcribed by the σ^s holoenzyme. Because an RpoS$^-$ mutant of Pf-5 did not produce detectable levels of pyrrolnitrin, we conclude that expression of structural genes for pyrrolnitrin production is under direct or indirect control by *rpoS*. In contrast to pyrrolnitrin, pyoluteorin and Phl were overproduced by the RpoS$^-$ mutant of Pf-5 in 523 medium. Because no intermediates are common to the proposed biosynthetic pathways of these antibiotics (34, 35), it is unlikely that production of pyrrolnitrin depletes a pool of precursors required for the biosynthesis of pyoluteorin and Phl. Instead, the *rpoS* gene may be required for transcription of a gene(s) encoding a protein that functions as a repressor of pyoluteorin and Phl biosynthesis in *P. fluorescens*. Alternatively, σ^s may serve as a negative regulator by competing for association with the RNA polymerase core with another sigma factor(s) required for production of Phl and pyoluteorin. Production of secondary metabolites and exoenzymes by *P. fluorescens* is likely to be controlled by a complex, multicomponent regulatory cascade, in which *rpoS*, *apdA*, and *gacA* are but three of many regulatory loci.

Like antibiotic production, biological control activities of Pf-5 were differentially affected by the *rpoS* mutation. Strain JL3985, which had a Tn*5* insertion in the *rpoS* gene, was more effective than the wild-type strain Pf-5 in suppression of postemergence damping-off of cucumber caused by *Pythium ultimum*. We attribute the enhanced disease suppression to the markedly increased production of pyoluteorin and Phl by the RpoS$^-$ mutant. In contrast, JL3985 was similar to Pf-5 in its capacity to suppress pre-emergence damping-off of cucumber caused by *Pythium ultimum*. In a previous study, mutants deficient in antibiotic production or those that overproduce pyoluteorin did not differ significantly from Pf-5 in suppressing pre-emergence damping-off of cucumber caused by *Pythium ultimum* (22). One explanation for these results is that concentrations of antibiotics that are adequate for fungal inhibition are not produced *in situ* by Pf-5 in time to protect seeds from infection by *Pythium ultimum*. Indeed, during the first 3 days after inoculation of seeds with Pf-5, *in situ* expression of pyoluteorin biosynthesis genes (*plt*) is low relative to expression by cells of Pf-5 grown in culture (36). It is nonetheless plausible that adequate concentrations of antibiotics are produced *in situ* by Pf-5 in time to protect emerged seedlings from infection by *Pythium ultimum*. The combined results of the present and past experiments are consistent with the hypothesis that the low levels of *in situ* pyoluteorin or Phl production by Pf-5 inhabiting seed or seedling surfaces limit suppression of *Pythium* damping-off. In contrast to the enhanced suppression of postemergence damping-off of cucumber, an *rpoS* mutation decreased the capacity of Pf-5 to suppress ascocarp formation by *Pyrenophora tritici-repentis* on wheat straw (10). Both poor survival on wheat straw over the course of an experiment (40 days) and the lack of pyrrolnitrin production presumably contributed to the diminished capacity of the RpoS$^-$ mutant to suppress *Pyrenophora tritici-repentis* (10).

Taken together, these data indicate that a mutation in a single regulatory locus such as *rpoS* can enhance or diminish the capacity of a bacterium to protect plants against infection.

It is generally accepted that bacterial cells exist for a substantial portion of their lives on plant surfaces in a nutrient-limited state. This concept is supported by recent studies in which the addition of exogenous nutrients increased the population size of *P. fluorescens* in the phyllosphere (37) or rhizosphere (38). It also is widely recognized that *Pseudomonas* spp. are exposed to multiple stresses in the varied microhabitats that they occupy in soil, the rhizosphere, and the phyllosphere (39, 40). The capacities to survive desiccation (39), osmotic stress (39), and oxidative stress (41) are traits determining fitness of plant-associated *Pseudomonas* spp. The capacities to survive exposure to these stresses are also characteristics of stationary-phase cells of *Pseudomonas* spp. (4, 5, 41), which express at least certain of these phenotypes under the control of *rpoS* (Figs. 2 and 3). Therefore, it is not surprising that an RpoS$^-$ mutant of Pf-5 is less persistent than the wild type on wheat straw residue (10). Similarly, when introduced onto cucumber seeds at high cell densities, *P. fluorescens* strain Pf-5 required a functional *rpoS* gene for optimal survival (Fig. 4*A*). In contrast, the RpoS$^-$ mutant did not differ from the parental strain either in exponential growth rate (10) or in the rate at which population size increased when Pf-5 was inoculated onto cucumber seeds at relatively low cell densities (Fig. 4*B*). The population size that was ultimately established by these cells defines a carrying capacity (Fig. 4*B*) that was exceeded by populations established by Pf-5 when inoculated at high cell densities (Fig. 4*A*). Therefore, populations of Pf-5 resulting from high cell-density inoculation probably encountered especially severe nutrient stress, in which a functional *rpoS* was essential for optimal survival. Populations of *Pseudomonas* spp. that are present in natural habitats are likely to encounter comparable periods of stress in which *rpoS* contributes to their fitness. The findings of this study indicate that *P. fluorescens* exists for a portion of its life on plant surfaces or residues in a physiological state in which RpoS is operative and influences the survival of the bacterium and its interactions with other microorganisms.

We thank Susan Carnegie for constructing pJEL01; Brian Nowak-Thompson for HPLC analysis of antibiotics produced by *P. fluorescens*; Anne-Marie Girard for sequence data; Gwyn Beattie, Steve Lindow, Walt Ream, and Virginia Stockwell for reviewing the manuscript; and Brian Nowak-Thompson, H. G. Floss, and Christoph Keel for providing authentic samples of antibiotics. This research was supported in part by a grant to A.S. from the North Atlantic Treaty Organization.

1. Thomashow, L. S. & Weller, D. M. (1995) in *Plant-Microbe Interactions*, eds. Stacey, G. & Keen, N. (Chapman and Hall, New York), Vol. 1, pp. 187–235.
2. Lam, S. T. & Gaffney, T. D. (1993) in *Biotechnology in Plant Disease Control*, ed. Chet, I. (Wiley–Liss, New York), pp. 291–320.
3. Leisinger, T. & Margraff, R. (1979) *Microbiol. Rev.* **43,** 422–442.
4. Givskov, M., Eberl, L., Møller, S., Poulsen, L. K. & Molin, S. (1994) *J. Bacteriol.* **176,** 7–14.
5. Klotz, M. G. (1993) *Can. J. Microbiol.* **39,** 948–957.
6. Morita, R. Y. (1993) in *Starvation in Bacteria*, ed. Kjelleberg, S. (Plenum, New York), pp. 1–23.
7. Kolter, R., Siegele, D. A. & Tormo, A. (1993) *Annu. Rev. Microbiol.* **47,** 855–874.
8. Howell, C. R. & Stipanovic, R. D. (1979) *Phytopathology* **69,** 480–482.
9. Howell, C. R. & Stipanovic, R. D. (1980) *Phytopathology* **70,** 712–715.
10. Pfender, W. F., Kraus, J. & Loper, J. E. (1993) *Phytopathology* **83,** 1223–1228.
11. Nowak-Thompson, B., Gould, S. J., Kraus, J. & Loper, J. E. (1994) *Can. J. Microbiol.* **40,** 1064–1066.
12. Keel, C., Schnider, U., Maurhofer, M., Voisard, C., Laville, J., Burger, U., Wirthner, P., Haas, D. & Défago, G. (1992) *Mol. Plant Microbe Interact.* **5,** 4–13.
13. Levy, E., Gough, F. J., Berlin, K. D., Guiana, P. W. & Smith, J. T. (1992) *Plant Pathol.* **41,** 335–341.
14. Corbell, N. & Loper, J. E. (1995) *J. Bacteriol.* **177,** 6230–6237.
15. Gaffney, T. D., Lam, S. T., Ligon, J., Gates, K., Frazelle, A., Di Maio, J., Hill, S., Goodwin, S., Torkewitz, N., Allshouse, A. M., Kempf, H.-J. & Becker, J. O. (1994) *Mol. Plant Microbe Interact.* **7,** 455–463.
16. Laville, J., Voisard, C., Keel, C., Maurhofer, M., Défago, G. & Haas, D. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 1562–1566.
17. Loewen, P. C. & Hengge-Aronis, R. (1994) *Annu. Rev. Microbiol.* **48,** 53–80.
18. Tanaka, K. & Takahashi, H. (1994) *Gene* **150,** 81–85.
19. Mulvey, M. R. & Loewen, P. C. (1989) *Nucleic Acids Res.* **17,** 9979–9991.
20. Itoh, Y., Watson, J. M., Haas, D. & Leisinger, T. (1984) *Plasmid* **11,** 206–220.
21. Chang, A. C. Y. & Cohen, S. N. (1978) *J. Bacteriol.* **134,** 1141–1156.
22. Kraus, J. & Loper, J. E. (1992) *Phytopathology* **82,** 264–271.
23. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), Ed. 2.
24. Rich, J. J. & Willis, D. K. (1990) *Nucleic Acids Res.* **18,** 6673–6676.
25. Kado, C. I. & Heskett, M. G. (1970) *Phytopathology* **60,** 969–976.
26. Takai, K. & Hayaishi, O. (1987) *Methods Enzymol.* **142,** 195–217.
27. Sarniguet, A., Lucas, P., Lucas, M. & Samson, R. (1992) *Plant Soil* **145,** 29–36.
28. Rich, J. J., Hirano, S. S. & Willis, D. K. (1992) *Appl. Environ. Microbiol.* **58,** 1440–1446.
29. McCann, M. P., Kidwell, J. P. & Matin, A. (1991) *J. Bacteriol.* **173,** 4188–4194.
30. Lonetto, M., Gribskov, M. & Gross, C. A. (1992) *J. Bacteriol.* **174,** 3843–3849.
31. Williams, M. D., Ouyang, T. X. & Flickinger, M. C. (1994) *Mol. Microbiol.* **11,** 1029–1043.
32. Nguyen, L. H., Jensen, D. B., Thompson, N. E., Gentry, D. R. & Burgess, R. R. (1993) *Biochemistry* **32,** 11112–11117.
33. Tanaka, K., Takayanagi, Y., Fujita, N., Ishihama, A. & Takahashi, H. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 3511–3515.
34. Cuppels, D. A., Howell, C. R., Stipanovic, R. D., Stoessl, A. & Stothers, J. B. (1986) *Z. Naturforsch.* **41,** 532–536.
35. Zhou, P., Mocek, U., Siesel, B. & Floss, H. G. (1992) *J. Basic Microbiol.* **32,** 209–214.
36. Kraus, J. & Loper, J. E. (1995) *Appl. Environ. Microbiol.* **61,** 849–854.
37. Wilson, M. & Lindow, S. E. (1994) *Appl. Environ. Microbiol.* **60,** 2232–2237.
38. Colbert, S. F., Schroth, M. N., Weinhold, A. R. & Hendson, M. (1993) *Appl. Environ. Microbiol.* **59,** 2064–2070.
39. Lindow, S. E., Anderson, G. & Beattie, G. A. (1993) *Appl. Environ. Microbiol.* **59,** 1593–1601.
40. van Elsas, J. D. & van Overbeek, L. S. (1993) in *Starvation in Bacteria*, ed. Kjelleberg, S. (Plenum, New York), pp. 55–79.
41. Katsuwon, J. & Anderson, A. J. (1990) *Appl. Environ. Microbiol.* **56,** 3576–3582.